Ras-Mediated Cleavage of a GTP Analogue by a Novel Mechanism

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The small guanosine triphosphate (GTP) binding protein Ras is involved in many cellular signal transduction processes leading to cell growth, differentiation and apoptosis. Mutations in ras *genes are found in a large number of human tumours. GTP hydrolysis, the process that normally leads to the transition of the Ras protein from the active (GTP-bound) form to the inactive (GDP-bound) form is impaired due to these oncogenic mutations. In contrast, the GTP analogue 3,4-diaminobenzophenone(DABP)-phosphoramidate-GTP, a substrate for GTP-binding proteins, enables switching to the inactive GDP form in both wild-type and oncogenic Ras. Here we show by HPLC, mass spectrometry and NMR spectroscopy that the mechanism of this DABP-GTPase reaction is different from the physiological GTPase reaction. The γ-phosphate group is not attacked by a nucleophilic water molecule, but rather by the aromatic amino group of the analogue, which leads to the generation of a stable cyclic diamidate product. These findings have potential implications for the development of anti-Ras drugs.*

KEYWORDS:

antitumor agents · hydrolases · Ras proteins · substrate-assisted catalysis

Introduction

Small guanosine triphosphate (GTP) binding proteins, of which Ras is the prototype, and heterotrimeric G-proteins cycle in a tightly regulated way between a GTP-bound conformation, in which they are active in a multitude of cellular signal transduction and other pathways, and an inactive, GDP-bound conformation. In the case of small GTP-binding proteins, the exchange of GDP for GTP is regulated by guanine nucleotide exchange factors (GEFs), and the hydrolysis of the bound GTP to GDP is promoted by GTPase-activating proteins (GAPs).[1]

Ras itself is a major regulator of many signal transduction processes such as cell growth, differentiation and apoptosis. It activates downstream targets such as the protein kinase Raf,[2] GEFs for the small GTP-binding protein Ral such as RalGEF[3] and the lipid kinase PI(3)-kinase.[4] Mutations in *ras* genes have been found in about 30% of human tumours, being particular prevalent in malignancies of the pancreas and the colon.[5] Oncogenic Ras proteins carry amino acid substitutions at one of the positions 61, 12 or (more rarely) 13, the biochemical consequence of which is a block in both the intrinsic and GAP-stimulated GTPase reaction. This results in a permanent "ON" signal of the Ras switch and thus contributes to tumour formation. Due to its importance in this process, the Ras protein is also widely recognised as an important anti-tumour target.

The GTP analogue 3,4-diaminobenzophenone-phosphoramidate of GTP (DABP-GTP, **I**; Figure 1) has been found to be cleaved by the α subunit of the heterotrimeric G-protein Gs (Gsα)[7] and Ras[8] with a rate similar to or faster than GTP. Studies with an oncogenic variant of Gsα showed that cleavage of the analogue, in contrast to the physiological GTPase reaction, does not require the catalytic glutamine at position 227 (corresponding to Gln 61 in Ras). More detailed studies with Ras showed that DABP-GTP is also efficiently cleaved by all oncogenic Ras proteins, with a rate acceleration of up to 1000-fold as compared to GTP hydrolysis. As in the case of the GTPase reaction, the inactive Ras·GDP complex is formed. The DABP-GTP reaction thus constitutes an attractive drug discovery pathway aimed at interfering with oncogenic Ras.

The deuterium isotope effect observed for the DABP-GTPase reaction[8] suggested that the mechanism is different from that of the GTPase reaction. The products are GDP[7, 8] and a phosphate group linked to the DABP moiety (DABP-P_i) of up to now unknown chemical structure. It was shown that the aromatic amino group of DABP-GTP is crucial for cleavage. For the reaction pathway, it has been proposed[9] that: a) The free amino group could act as a general base to activate a nucleophilic water molecule, b) the amino group itself could act as a nucleophile or c) it could enable the phosphoramidate nitrogen to act as a general base for an attacking water molecule, d) the transition state could be stabilised by a hydrogen bond between the free amino group and an attacking

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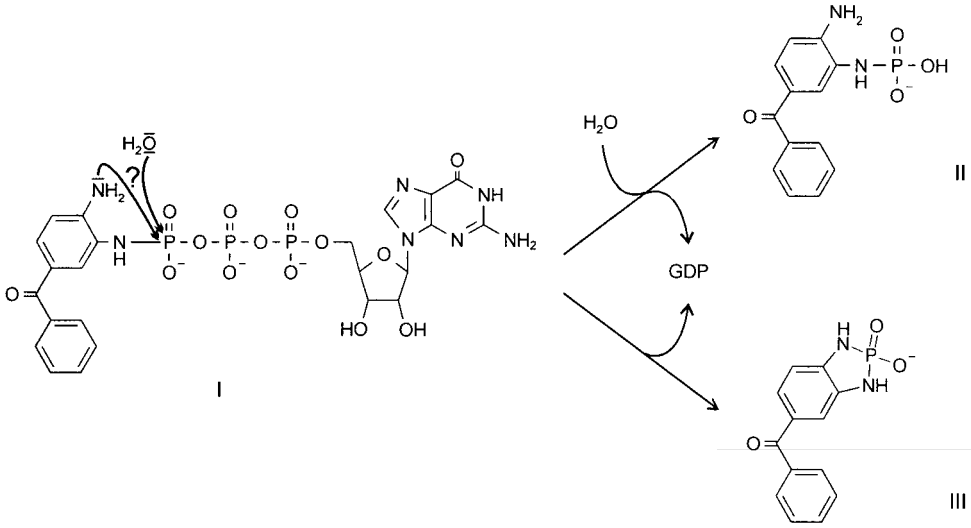

Figure 1. *The structure of DABP-GTP (**I**) and two possible structures for the DABP-P_i reaction product, a monoamidate (**II**) obtained through hydrolysis or a cyclic phosphodiamidate (**III**) obtained through direct attack of the aromatic amino group on the γ-phosphate group.*

water molecule. The mechanisms a), c) and d) involving a nucleophilic water molecule would yield a DABP-P_i molecule in which oxygen from solvent water is incorporated and which has only one covalent P–N bond (**II** in Figure 1). On the other hand, a nucleophilic attack of the amino group would lead, at least transiently, to a product with two P–N bonds (**III** in Figure 1). Here, we have analysed the mechanism of DABP-GTP cleavage. It is concluded that, contrary to the models favoured so far,[7–9] the cleavage of DABP-GTP differs fundamentally from GTP hydrolysis because it occurs through direct attack of the aromatic amino group rather than hydrolysis and because a stable cyclic reaction product is formed.

[*] *Members of the Editorial Advisory Board will be introduced to the readers with their first manuscript.*

Experimental Section

Proteins and nucleotides: Recombinant H-Ras variants were expressed in and isolated from *Escherichia coli* as described previously.[6] Nucleotide-free protein was prepared essentially as published by John et al.[10] DABP-GTP was synthesised as described previously[7] and the reaction product was purified by ion-exchange chromatography on Q-sepharose (Pharmacia) using elution with a concentration gradient of 100 → 1000 mM triethylammonium hydrogen carbonate buffer. The pooled fractions of DABP-GTP were lyophilised.

Kinetics of DABP-GTP cleavage: The reaction buffer was 30 mM Tris/HCl (pH 7.5), 3 mM dithioerythritol, 5 mM KH_2PO_4/K_2HPO_4 (KP_i; pH 7.5), with either 5 mM $MgCl_2$ or 5 mM ethylenediaminetetraacetic acid (EDTA), in normal water or $[^{18}O]H_2O$. The DABP-GTP concentration was typically 100 μM. For the Ras-catalysed reactions, nucleotide-free protein was added in an excess of ca. 20%. The samples were incubated at 30 °C. Aliquots taken at different time points were shock-frozen in liquid nitrogen to stop the reaction. The samples were then quickly thawed and analysed by HPLC on an RP-C18 column under isocratic conditions (100 mM KP_i (pH 6.5), 10 mM tetrabutylammonium bromide, 25% acetonitrile). From the peak areas, the DABP-GTP/(DABP-GTP + DABP-P_i) ratio was calculated and fitted to a single-exponential curve. The DABP-P_i peak rather than the GDP peak was chosen for quantification due to overlap of the latter with buffer peaks.

NMR spectroscopy: For NMR spectroscopic measurements, DABP-GTP was cleaved either in 50 mM Tris/HCl (pH 7.5) buffer containing a three- to fivefold excess of $MgCl_2$ or in a protein-catalysed reaction as described above. DABP-P_i was purified either on a Resource Q ion-exchange column (Pharmacia) with a triethylammonium acetate gradient (50 → 1000 mM) or on a Q sepharose column using a triethylammonium hydrogen carbonate gradient (500 → 1500 mM). Pooled fractions of the product were lyophilised and dissolved in a buffer containing 50 mM Tris/HCl (pH 7.5) and 50% D_2O. NMR spectra were recorded on Bruker DRX500 and Varian Inova 600 spectrometers. To shift the pH to 8.5, NaOH was carefully added. Phosphoric acid and tetramethylsilane were added as standards in the ^{31}P and ^{13}C NMR spectroscopic measurements, respectively.

Mass spectrometry: Negative-mode ESI spectra were measured on a Finnigan MAT LCQ mass spectrometer coupled to a Hewlett–Packard 1100 unit. For one spectrum, 10 μL of a solution containing ca. 100 μM DABP-P_i were used.

Results and Discussion

DABP-GTP cleavage

The time course of the reaction of Ras·DABP-GTP can conveniently be followed by reverse-phase HPLC as described previously.[8, 11] Elution profiles of DABP-GTP and the reaction products after complete RasQ61A-mediated cleavage are shown in Figures 2a, b. Two reaction products can be identified, one of which is GDP, as demonstrated by a GDP control sample, whereas the other is tentatively labelled as DABP-P_i. The compound can be purified by ion-exchange chromatography (Figure 2c). To analyse its nature by NMR spectroscopy we needed to conveniently prepare large amounts of DABP-P_i. We thus tested whether the DABP-GTPase reaction is absolutely dependent on the presence of protein.

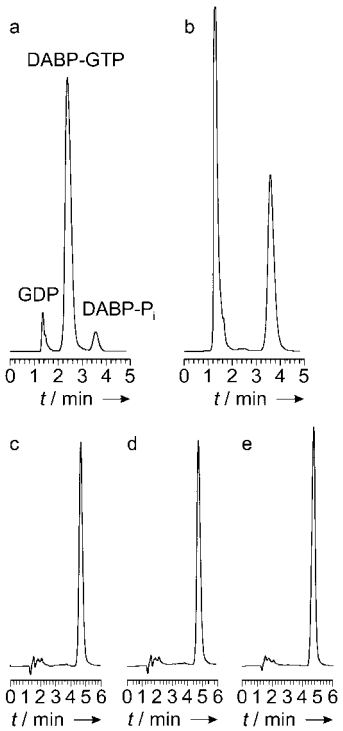

Figure 2. Reverse-phase HPLC analysis of the starting material and the reaction products. Elution profiles of a) the DABP-GTP educt, indicating the position of the corresponding compound; b) the products of DABP-GTP cleavage with RasQ61A; c) purified DABP-P_i produced with RasG12V; d) purified DABP-P_i produced without Ras. e) Co-elution of the two products. Note that in c) – d) a different column was used than in a) and b).

The DABP-GTPase rates of several Ras oncoproteins in the presence of Mg^{2+} have been determined by HPLC.[8] In the absence of both Ras and metal ions, DABP-GTP is very stable and cleaved only at very low rates, with a half-life of 46 h (Figure 3, Table 1). In the presence of 5 mM Mg^{2+} the reaction becomes 38-fold faster, with a half-life of 74 min, which is still much slower than in the presence of Ras. This suggests that the electron-withdrawing potential of the metal ion polarises the γ-phosphate group so that a nucleophilic attack on the electron-deficient phosphorus atom is facilitated and/or the metal ion stabilises the negative charge on the γ-phosphate group in the ground and/or transition state. Figure 2d shows the elution profile of the purified DABP-P_i produced without protein. The protein-free reaction rate is independent of the DABP-GTP concentration in the range of 5 – 100 μM (data not shown), indicating first-order kinetics. The presence of Ras leads to an additional increase in the reaction rate ranging from 14-fold (RasG13A, data taken from Ahmadian et al.[8]) to 181-fold (RasG12V), but the protein-induced reaction is also dependent on the presence of Mg^{2+} (Table 1). The large rate increase on binding of DABP-GTP to Ras suggests that fixation and orientation of the nucleotide analogue on the protein surface is favourable for the cleavage reaction. The rate enhancement is strongly dependent on the nature of the Ras protein, oncogenic mutants with large hydrophobic residues in the 12-position showing the strongest effect. We postulate that this is due to the differences in the conformation of the DABP moiety of the substrate on the protein surface. In X-ray crystal structures of RasG12V and RasG12P complexed to the non-cleavable DABP-GTP analogue DABP-GPPNHP, differences in the structure around the GTP analogue were indeed observed.[8]

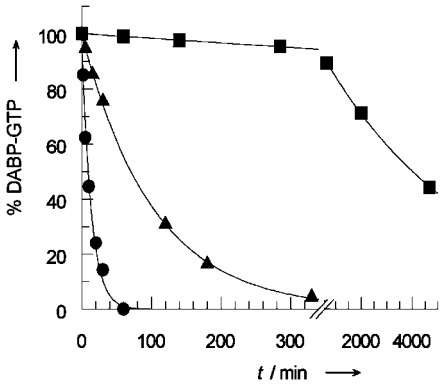

Figure 3. Time course of different DABP-GTP cleavage reactions. DABP-GTP cleavage was measured in the absence of both protein and Mg^{2+} (■), without protein in the presence of Mg^{2+} (▲), and in the presence of RasG12V without Mg^{2+} (●). The rate was followed by taking aliquots of the reaction mixture at the indicated time points and analysing them by HPLC. Concentrations of educt and products were obtained from a quantitative analysis of the elution peaks, and the rates were calculated as described in the Experimental Section.

Table 1. Cleavage rates and corresponding half-lives of DABP-GTP.

RasG12V	Mg^{2+}	Mn^{2+}	rate [min^{-1}]	relative rate[a]	half-life [min]
+	+	–	1.7	1	0.41
+	–	+	1.0	0.59	0.69
+	–	–	5.5×10^{-2}	0.032	13
–	+	–	9.4×10^{-3}	0.0055	74
–	–	+	9.1×10^{-2}	0.054	7.6
–	–	–	2.5×10^{-4}	0.00015	2800

[a] The rates are compared to the rate obtained with RasG12V in the presence of Mg^{2+}.

Nature of the metal ion

Mg^{2+} is complexed to nucleotide-loaded Ras in a stoichiometric ratio. The intrinsic GTPase activity of wild-type Ras was analysed

by Schweins et al.[12] with a linear free energy relationship approach. The rate is accelerated 4.4-fold if the Mg^{2+} ion is replaced by the transition metal ion Mn^{2+}. This is due to an increase in the pK_a of the γ-phosphate group of GTP, which serves as the general base in the hydrolysis reaction. Mn^{2+} also accelerates the GTP hydrolysis of RasG12V (data not shown). However, in the case of the DABP-GTPase of RasG12V, one does not observe a rate acceleration by Mn^{2+} ions. In contrast, the protein-free DABP-GTP cleavage in the presence of Mn^{2+} is ten times faster than with Mg^{2+}, for reasons that are not obvious at the present moment, but may be related to the different stabilities of metal ion – nucleotide complexes.

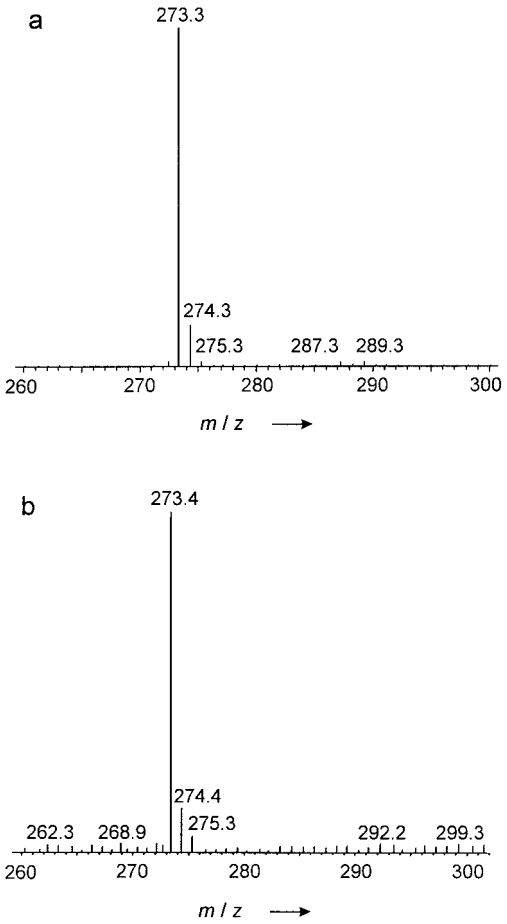

Figure 4. *Electrospray ionisation mass spectrometric analysis (negative mode) of the DABP-GTP cleavage product DABP-P_i obtained from the reaction in a) normal or b) ^{18}O-labelled water.*

Identification of DABP-P_i

To further clarify whether the presence of Ras has an influence on the products formed, DABP-GTP cleavage was performed with Mg^{2+} in the presence or absence of RasG12V. The purified DABP-P_i products (ca. 95% purity) in both cases eluted with an identical retention time in reverse-phase HPLC, and coeluted when injected together (Figures 2c – e). The elution time of the side product in DABP-GTP preparations is also identical (Figure 2a), indicating that no apparent modification took place during the purification. This, together with ^{31}P NMR spectroscopic data (see below), suggested that the products were identical.

We then used electrospray ionisation mass spectrometry for the analysis of DABP-P_i produced in the presence of Mg^{2+}. One would expect an *m/z* value of 291.2 for the monoamidate form of DABP-P_i (compound **II**, Figure 1), whereas it would be 273.2 for the cyclic diamidate form (compound **III**). Both the reaction products in the presence or absence of Ras showed the same major peak with a mass of $m/z = 273.3 \pm 0.1$ (Figure 4a). To make sure that the same product is obtaineed in the absence of Gln61, which is essential for GTP hydrolysis, the same analysis was performed with RasQ61A as a representative of the other class of oncogenic mutants. Mass spectrometry showed the same result (data not shown), indicating that cleavage does indeed occur by an intramolecular attack of the amino group on the γ-phosphate group. An identical mass was also found in the presence of Mn^{2+}. In these experiments, no peak corresponding to the monoamidate form was detected.

To exclude the possibility that the cyclic product is not formed by aminolysis but through cyclisation of a hypothetical monoamidate intermediate arising through hydrolysis, experiments in buffer containing $[^{18}O]H_2O$ instead of normal water were performed. Once again, the same mass indicative of a cyclic product was found (Figure 4). A transiently formed monoamidate would contain one ^{18}O atom, so that in $^2/_3$ of the cases one would expect a diamidate with an *m/z* value higher by 2 units. A second population was not observed. The identical mass when using ^{18}O-labelled and normal water shows that no atoms from the solvent are incorporated into the product. The mass spectrometric analysis thus constitutes a major argument in favour of a 5-ring product formation due to nucleophilic attack of the amino group on the γ-phosphorus atom.

NMR analysis of DABP-P_i

Analysis by ^{31}P NMR spectroscopy shows singulets at $\delta = 20.4$ for DABP-P_i obtained with and without Ras (Figure 5). This lies within the expected range for diamidates of phosphoric acid such as *N*,*N*,*N*′,*N*′-tetramethylphosphorodiamidic acid *O*-methyl ester ($\delta = 19$),[17] and is an additional indication that both products are identical.

To further characterise the product of the reaction, ^1H and ^{13}C 1D and 2D NMR experiments were performed. As no difference in the product formed with different Ras variants and without protein was detectable by HPLC, mass spectrometry and ^{31}P NMR, purified DABP-P_i produced in the absence of protein was used. $^1H/^{13}C$ direct and long-range correlation, and ^1H and ^{13}C 1D NMR spectra were recorded at pH 7.5 in order to verify the cyclic structure of DABP-P_i (Figure 6a). In a symmetrical molecule like the cyclic product, the two carbon atoms C_f and C_c (see Figure 6c) are expected to have a more similar chemical environment than in an asymmetrical structure like the monoamidate. The chemical shifts of these two nuclei are indeed similar ($\delta = 108.1$ and 110.1, respectively). Due to phosphorus coupling, the C_f peak is a doublet ($^3J_{PNCC} = 11.8$ Hz) like the C_c peak, which has a similar coupling constant ($^3J_{PNCC} = 12.5$ Hz). In

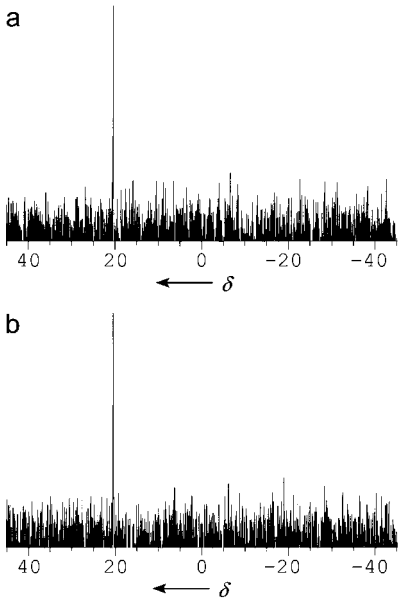

Figure 5. *^{31}P NMR spectrum of DABP-P_i produced a) with RasG12V and b) in the absence of Ras.*

the postulated 5-ring structure of DABP-P_i, either of these atoms and the phosphorus atom are separated by three covalent bonds. In contrast, the signals for the two carbon atoms one additional bond away from the phosphorus atom, C_e and C_d, are observed as singulets. If DABP-P_i was a monoamidate and thus had only one P—N bond, the distance P – C_f would be four bonds. In the case of C_d, a four-bond distance does not lead to a split of the signal into a doublet. At pH 7.5, no more than nine ^{13}C peaks are readily observed for the DABP-P_i molecule in the region of $\delta = 108 - 140$ (the carbonyl C_g signal appears at $\delta = 199.8$), although there are ten different spin systems. However, the long-range correlation at 500 MHz (pH 7.5) indicates an overlay of two signals at $\delta = 132.3$ (C_b and C_l), as no coupling between H_f and a C atom in the other aromatic ring is possible. We assumed that the C_b peak is a doublet like that of C_a. A long-range correlation spectrum was obtained at 600 MHz and pH 8.5 (Figure 6b). The separation of the C_b and C_l peaks was improved, but not complete. An additional correlation to H_c was observed as a further indication that the C_b peak is partially masked by the C_l peak. Assuming a partial overlay of a C_b doublet, the coupling constant $^2J_{PNC}$ would be about 9 Hz. For the adjacent carbon C_a one finds $^2J_{PNC} = 11.8$ Hz. Due to the increased sensitivity at 600 MHz, additional correlation signals of the unsubstituted phenyl ring were detected. The small ^{1}H peaks marked with an asterisk most probably appeared through decomposition of DABP-P_i at $-20\,^{\circ}$C, as the 600-MHz spectra were recorded after storage of the NMR sample for several months at pH 8.5. Taken together, the NMR spectroscopic data support a cyclic structure of DABP-P_i arising from a direct nucleophilic attack of the amino group on the γ-phosphorus atom.

Stability of DABP-P_i

At pH 7.5, no substantial degradation of DABP-P_i was detectable in 2D NMR experiments after six days at room temperature, and at pH 8.5 it appeared stable enough for recording several NMR spectra. It was anticipated that in acidic or basic solution the 5-ring is broken. Acid- and base-catalysed hydrolysis of DABP-P_i did not lead to the monoamidate form, however. A product was formed that had the same mass and HPLC elution properties as diaminobenzophenone (data not shown). Thus, it appears that, under these conditions, once the ring is opened, hydrolysis rapidly proceeds to completion.

Implications for anti-Ras drugs

To switch off GTP-binding proteins, bound GTP has to be hydrolysed to GDP. If GTP hydrolysis is impaired, these proteins are permanently switched on. Mutated forms of Ras, Gsα and

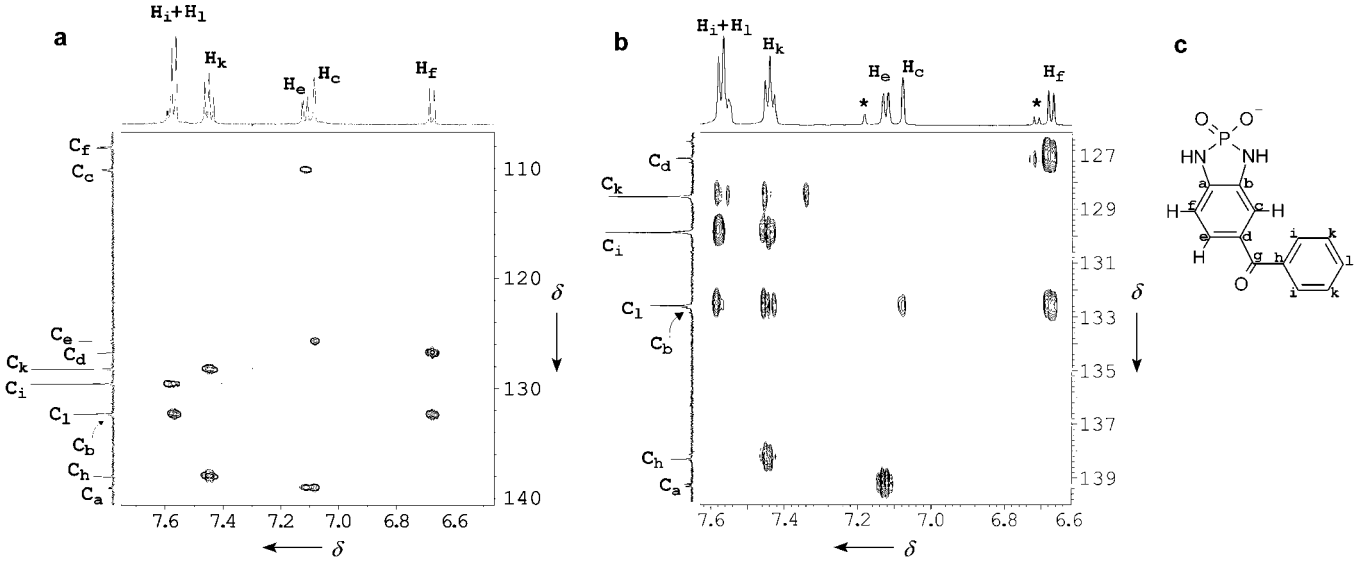

Figure 6. *Long-range ^{13}C/^{1}H correlation spectrum at a) pH 7.5, 500 MHz, b) pH 8.5, 600 MHz. c) Structure of cyclic DABP-P_i.*

Giα act as oncogenes in human tumours,[13] and activated forms of Rho/Rac and Cdc42 have also been shown to contribute to transformation of rodent fibroblast cell lines.[14] GTP hydrolysis on Ras occurs through a direct in-line nucleophilic attack of water,[15] where GTP serves as the general base necessary for activation of the attacking water molecule,[11] and the same mechanism has also been proposed for other GTP-binding proteins. The inability of oncogenic Ras proteins to hydrolyse GTP is either due to the missing catalytic Gln residue or to steric interference of P-loop mutants with the transition-state geometry.[16] The GTP analogue DABP-GTP bypasses such defects and allows inactivation of oncogenic GTP-binding proteins.[7, 8] This has stimulated the idea of generating a small anticancer drug capable of entering the active site of oncogenic GTP-bound Ras and effecting the transition to the GDP form. It was proposed[8] that the chemistry of DABP-GTP as well as knowledge of the intrinsic and the GAP-catalysed GTP hydrolysis mechanisms[11, 16] could serve as guidelines in the search for such compounds.

Here we show that, contrary to previous assumptions,[7–9] the DABP-GTPase mechanism is not related to the intrinsic and GAP-accelerated GTP hydrolysis by Ras, and does not involve hydrolysis, but rather an aminolysis reaction. The deuterium isotope effect of DABP-GTP cleavage[8] might be explained by a slow deprotonation after ring formation, although we cannot exclude other possibilites. The direct nucleophilic attack of the aromatic amino group on the γ-phosphate group leads to formation of GDP and of a cyclic phosphodiamidate whose identity has been verified by mass spectrometry. The NMR measurements also confirmed a cyclic structure of DABP-P_i, as the ^{13}C NMR spectra cannot be easily reconciled with a non-cyclic product. HPLC, ^{31}P NMR spectroscopy and mass spectrometry indicated that an identical product is formed with and without Ras. Thus, the roles of the catalytic glutamine residue in GTP-binding proteins and the aromatic amino group in DABP-GTP are different, and conclusions on the GTPase mechanism cannot be drawn from findings obtained with DABP-GTP or vice versa. The different mechanism may have fundamental implications in the search for an anti-Ras drug based on the idea of stimulating the GTPase reaction of oncogenic Ras: It shows that oncogenic Ras is not per se resistant to transition to its inactive GDP-bound form and that suitably located chemically reactive groups on the surface of the protein might allow to turn off the molecular switch locked in its "ON" position. From the evidence presented here and in our previous report[8] such a chemically active group would in principle be independent of which type of oncogenic mutation is present in the tumour.

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